JP-A-2002-272463

(54) [Title of the Invention]

Method for judging type of single nucleotide polymorphism

(57) [Abstract]

[Target] To provide a method capable of judging a type of single nucleotide substitution in a simple manner and in a short time.

[Solving Means]

A method of judging a type of single nucleotide substitution including a step of preparing a polynucleotide which is formed by a hybridization of a primer with a target polynucleotide having a single nucleotide polymorph site in n-position and in which a nucleotide positioned at a 3'-end of the primer hybridizes with a nucleotide in (n+1)-position of the target polynuclotide; a step of executing an extending reaction by a modified nucleotide which is complementary to a nucleotide species to be detected, present in the polymorph site and which is so modified and marked as to terminate a further extending reaction; a step of measuring a positional change, as a function of time, in a minute space of the marking; and a step of analyzing the result of measurement of the preceding step with a fluorescence correlation analysis to determine whether the marked nucleotide is fetched in the polynucleotide, thereby judging a type of the single-nucleotide polymorphism.

***** ***** *****

[Claims]

[Claim 1]

A method of judging a type of single-nucleotide polymorphism comprising:

a step of preparing a polynucleotide which is formed by a hybridization of a primer with a target polynucleotide having a single nucleotide polymorph site in an n-position (n being an integer of 1 or larger) and in which a nucleotide positioned at a 3'-end of said primer hybridizes so as to correspond to a nucleotide in an (n+1)-position of the target polynuclotide; a step of executing an extending reaction by mixing, with the polynucleotide prepared in the preceding step, a modified nucleotide which is complementary to a nucleotide species to be detected, among the nucleotide species that can be present in said single nucleotide polymorph site and which is marked with a traceable marker and so modified as to terminate a further extending reaction; a step of measuring a positional change, as a function of time, in a minute space of said marker; and a step of analyzing the result of measurement of the preceding step with a fluorescence correlation analysis to determine whether the marked nucleotide, complementary to the nucleotide to be detected, is fetched in said polynucleotide in said extending reaction step, thereby judging a type of the single-nucleotide polymorphism.

[Detailed Description of the Invention]

[0001]

[Technical Field to which the Invention Belongs]

The present invention relates to a method for judging a type of single

part.

[0004]

Also there has been conceived a method of detection by mass spectroscopy, utilizing such primer extension method. Tang et al. have developed a method called PROBE (primer oligo base extension) (Proc. Natl. Acad. Sci. USA, 96: 10016 - 10020). In this method, a DNA of a length of about 100 bp including an SNP site is amplified by PCR, purified by desalting, subjected to a chemical modification at 5'-end such as a biotination or a thiolation, and immobilized on a silicon chip bearing lattice-shaped wells. After immobilization and washing, a primer extension reaction is executed on the chip, utilizing three dNTPs and a ddNTP. After the reaction, the chip is washed, then a TOP MASS matrix solution is added to crystallize the sample, and finally MALDI (matrix assisted laser desorption ionization) mass spectroscopy is executed to identify the base species extended by the extension reaction, namely the base species of the single-base mutation site. Such primer extension reaction is to directly detect a base immediately close to the 3'-terminal of the primer, and is a method effective for detecting a single-base mutation. Also the method by MALDI TOF provides an effect of not requiring a labeling on the base species. However, such technologies require to immobilize the target nucleic acid molecules on a certain solid surface in order to execute a primer extending reaction, and to execute a hybridization and an extending reaction of the primer with the immobilized nucleic acid molecules, thus involving drawbacks of a significantly lowered reaction efficiency and complex operations.

[0005]

In the present specification, "single nucleotide polymorphism" means a genetic polymorphism based on a single-base mutation.

[0008]

[Embodiments of the Invention]

The present invention provides a method for judging a type of single nucleotide polymorphism.

[0009]

In the present specification, "judging a type of single nucleotide polymorphism" means to estimate or to determine species of a nucleotide, present in the site showing a single nucleotide mutation (single nucleotide polymorphism site). Therefore, the method of the present invention allows, for example when adenine, guanine or cytosine is known to be present in the single nucleotide polymorphism site, to determine the nucleotide in such site as adenine or other than adenine.

[0010]

The target polynucleotide including a single nucleotide polymorphism site is a single-chain polynucleotide in the following examples, but it is not restricted to a single chain and a duplex-chain polynucleoide may also be utilized.

[0011]

In the following, the present invention will be further clarified by examples, but the following examples do not intend, in any meaning, to restrict the scope of the present invention.

[0012] [Example 1]

The present example explains, with reference to Fig. 1, a method of

The marker may be any arbitrary marker that is traceable, but a preferable marker may be a light-emitting marker, particularly a fluorescent marker.

[0018]

Fig. 1 shows, as an example, a case where the nucleotide type to be detected is adenine, so that the nucleic acid base contained in the marked modified nucleotide is thymine.

[0019]

After the addition of the marked modified nucleotide 15, an extending reaction is executed. The extending reaction may be executed utilizing an enzyme, such as DNA polymerase.

[0020]

The marked modified nucleotide 15 is so modified as to terminate a further extending reaction. "Modification so as to terminate a further extending reaction" means that the modified nucleotide 15 itself is so modified, in the nucleic acid extending reaction, as to be added to the primer thereby extending the primer, but as not to cause a subsequent extending reaction. Therefore, utilizing the modified nucleotide 15, the extending reaction is terminated after an extension by one base. Such modified nucleotide may be dideoxynucleotide that is often utilized for example in the field of base sequence determination of nucleic acid, but is not restricted thereto.

[0021]

As the extending reaction is terminated after an extension by one base, the marked modified nucleotide 15 is added to the 3'-end of the primer 12 constituting the partially duplex-chain polynucleotide 14, only when the

2, by:

1. emitting, from a laser light generating apparatus 22, a laser light as an excitation light;

2. condensing the laser light after passing a filter 23, and irradiating a point of the sample with the laser light by a dichroic mirror 24;

3. exciting a fluorescent substance in the sample with the laser light, thereby causing a light emission;

4. passing only a fluorescence, emitted from the fluorescent substance at the focal point in the sample, through a pinhole 25 and amplifying the fluorescence by a photomultiplier (PMT) 26; and

5. analyzing the amplified fluorescence by a data processing apparatus 27 and displaying a result on a display apparatus 28.

[0027]

As a light source for the confocal microscope, for example an argon ion laser may be employed, but a krypton argon ion laser, a helium-neon laser, or a helium-cadmium laser may also be employed according to the type of the fluorescent substance.

[0028]

Such detection method, being adapted to detect only the fluorescence emitted from the target present in the minute space, has an extremely low background and shows a significantly higher sensitivity, in comparison with an ordinary fluorescence detection.

[0029]

"Measurement of positional change of the marker as a function of time" is generally executed in an order of from a millisecond to a minute, and most

3. An expected value of a product of fluorescence intensities I(t) and I(t+τ) at two different times, to obtain a self correlation function $G(\tau) = \langle I(t)\, I(t+\tau)\rangle$;

4. The self correlation function obtained in 3 is analyzed, utilizing a following equation 1:

[mathematic 1]

$$G(t)=1+\frac{1}{N}\left[\left\{\frac{1-y}{1+\frac{t}{\tau\,small}}\sqrt{\frac{1}{1+S^2\cdot\frac{t}{\tau\,small}}}\right\}+\left\{\frac{y}{1+\frac{t}{\tau\,large}}\sqrt{\frac{1}{1+S^2\cdot\frac{t}{\tau\,large}}}\right\}\right]$$

(wherein:

N: average number of fluorescent molecules;

$\tau_{small} = wo^{2/4}D_{small}$: parallel diffusion time of nucleic acid of small size;

$\tau_{large} = wo^{2/4}D_{large}$: parallel diffusion time of nucleic acid of large size;

y: proportion of a repeating sequence of a large repeating number;

S: wo/zo

(wo being a diameter of a detection area; 2zo being a length of the area; D_{small} and D_{large} being parallel diffusion constants respectively of nucleic acid of a small size and nucleic acid of a large size)).

[0034]

For the data analysis by FCS, there may be employed a computer program "FCS" commercialized from Evotec BioSystems Inc. An operation time of the steps from 1 to 4 may be less than 10 seconds per a sample.

[0035]

Concept of such analysis will be made clearer by making reference to Fig. 3. More specifically, in case of a smaller size, I(t) has a higher frequency because of a larger velocity of Brownian movement. In case of a larger size, I(t) has a lower frequency because of a smaller velocity of Brownian movement.

[0036]

proportion, but the marked modified nucleotide 15 is present in a larger amount than the target single-chain polynucleotide 11. Therefore, a curve of a shape as indicated by 19 is often obtained between the curves 17 and 18.

[0041]

When the marked modified nucleotide 15 is present in excess with respect to the target single-chain polynucleotide 11, the modified nucleotide 15 incorporated in the target single-chain polynucleotide 11 represents only a very small amount. Therefore, a curve obtained in such case may be undistinguishable from the curve 17, and may be judged that the modified nucleotide 15 has not been incorporated in the target single-chain polynucleotide 11. Therefore, an amount of the modified nucleotide 15 that has not been incorporated in the target single-chain polynucleotide 11 is preferably a half or less of a total amount of the modified nucleotide 15.

[0042]

On the other hand, as an excessively small amount of the marked modified nucleotide 15 extremely retards the progress of the extending reaction, it is preferable to utilize the marked modified nucleotide 15 in such an amount that the extending reaction can proceed appropriately.

[0043]

As explained above, the method of the present example allows to judge, in simple and prompt manner, whether the nucleotide type to be detected is present in the single nucleotide polymorphism site.

[0044]

The present example has been explained by a case, as an example, where a site is already made clear as a site having the single nucleotide polymorphism,

[0048]

In the example shown in Fig. 4, adenine (A) or guanine (G) may be present at the single nucleotide polymorphism site 43.

[0049]

After the preparation of the partially duplex-chain polynucleotide 44, marked nucleotides 45, 46, which are complementary to the nucleotide types that can be present in the single nucleotide polymorphism site, are mixed with the partially duplex-chain polynucleotide 43. In the example shown in Fig. 4, the marked nucleotide 45 is dideoxythymidine, and the marked nucleotide 46 is dideoxyguanosine. In order to distinguish both marked nucleotides, it is preferable to mark the marked nucleotide 45 and the marked nucleotide 46 with different substances.

[0050]

In the case that adenine is present in the single nucleotide polymorphism site 43 of the target single-chain polynucleotide 41, the marked nucleotide 45 is added to an end of the primer 42. Also in the case that cytosine is present in the single nucleotide polymorphism site 43 of the target single-chain polynucleotide 41, the marked nucleotide 46 is added to an end of the primer 42.

[0051]

After the extending reaction, a positional change of the marker in a minute space, in the marked nucleotide, is measured as a function of time. The measuring method is the same as described in Example 1, and a curve 47 is obtained when the marked nucleotide is not incorporated, while a curve 48 is obtained when the marked nucleotide is incorporated.

[0052]

18 curve

19 curve

21 confocal microscope

22 laser generating apparatus

23 filter

24 dichroic mirror

25 pinhole

26 photomultiplier

27 data processing apparatus

28 display apparatus

41 target single-chain polynucleotide

42 primer

43 single nucleotide polymorphism site

44 partially duplex-chain polynucleotide

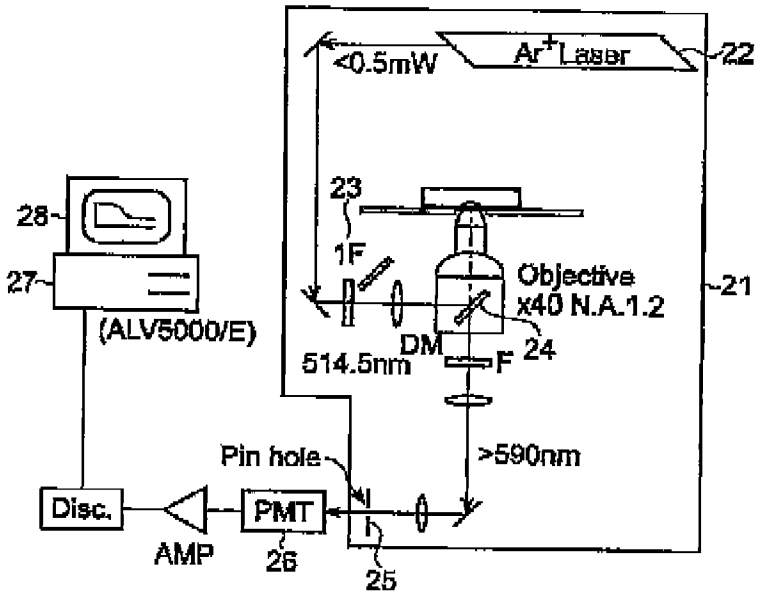
45 marked nucleotide

46 marked nucleotide

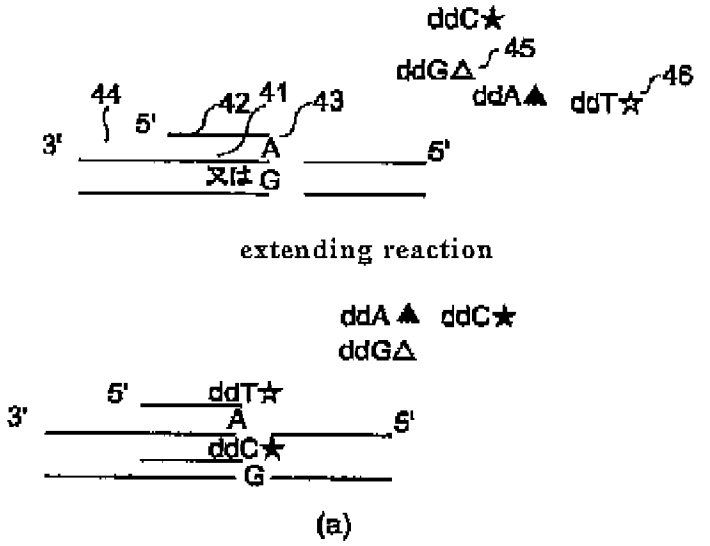
47 curve

48 curve

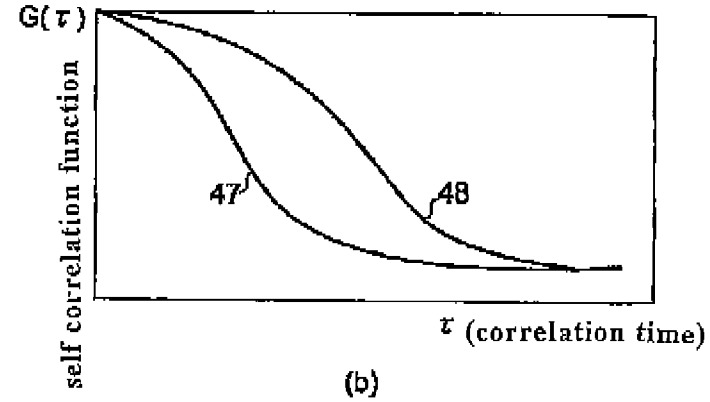
[Fig. 2]


<0.5mW
Ar+Laser
22
23
1F
Objective
x40 N.A.1.2
21
28
27
(ALV5000/E)
514.5nm
DM
F
24
Pin hole
>590nm
Disc.
AMP
PMT
26
25

[Fig. 4]


ddC★
ddG△
45
ddA▲
ddT☆
46
44
5'
42
41
43
3'
A
又は G
5'
extending reaction
ddA▲ ddC★
ddG△
5'
ddT☆
3'
A
5'
ddC★
G

(a)


G(τ)
self correlation function
47
48
τ (correlation time)

(b)